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Award Number: W81XWH-12-1-0125

TITLE: Zebrafish Model of NF1 for Structure-Function Analysis, Mechanisms of Glial Tumorigenesis, and Chemical Biology

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CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute

REPORT DATE: May 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
May 2014	Annual	1 May 2013 – 30 April 2014

4. TITLE AND SUBTITLE

Zebrafish Model of NF1 for Structure-Function Analysis, Mechanisms of Glial Tumorigenesis, and Chemical Biology

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0125

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dana-Farber Cancer Institute
Office of Grants and Contracts
Boston, MA 02215-5450

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the past year, we have continued to further our studies towards meeting the goals set forth in the funded proposal. An exciting phenotype has been identified that is relevant to the human NF1 disease and to the potential development of meaningful therapies. In collaboration with the Granato lab at Penn, the Epstein lab has discovered striking learning and memory defects in mutant fish. In the Look lab, the zebrafish models of the NF1 tumor suppressor linked cancers of MPNST and glioma have been completely updated to make them optimal for the structure function studies. Three different strategies are being applied to further accelerate the onset of these tumors to increase the precision of the structure function studies to elucidate the mechanism of tumor suppression by NF1. We have shipped our NF1 zebrafish lines to colleagues in the NF1 and zebrafish communities. These genetically engineered lines will be excellent candidates for use in screening small molecules and for use in performing genetic screens to identify modulators of this important disease.

15. SUBJECT TERMS
Neurofibromatosis; learning and memory; glioma; MPNST

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common genetic disorder caused by mutations in the *NF1* gene. Patients exhibit pigmentation abnormalities, learning disorders, bone abnormalities and multiple benign and malignant tumors, including tumors of neural crest origin such as glioma and malignant peripheral nerve sheath tumors (MPNSTs). The *NF1* gene encodes neurofibromin, a protein of over 4,000 amino acids. So far, only a 360 aa region of neurofibromin has been functionally characterized as a GAP-related domain (GRD) which is capable of accelerating the hydrolysis of GTP bound to Ras, thus down-regulating Ras activity. Additional important functions of neurofibromin are yet to be discovered. The purpose of this study is to exploit a new vertebrate animal model of NF1. We capitalize on the advantages of zebrafish as a model system to address pressing questions relevant to the generation of new and effective therapies for NF1. The zebrafish model system will allow the Epstein lab in Aim 1 to perform rapid in vivo rescue "structure-function" experiments using wild type and mutant NF1 genes, as well as constructs expressing portions of the NF1 protein, in order to determine the functional domains of neurofibromin that have not been previously identified or evaluated. These structure-function mutants will also be used by the Look lab in Aim 2 to understand the role of nf1 in glioma and MPNST tumorigenesis, which will reveal clinically relevant mechanistic information about neurofibromin function and identify unrecognized functional domains of neurofibromin appropriate for therapeutic targeting.

BODY

Aim 1: To fully characterize *nf1a/b* compound null zebrafish and to perform structure-function studies of Nf1 *in vivo* by performing rescue experiments in *zn1a/b* loss-of function zebrafish to determine which aspects of the mutant phenotype are regulated by GAP and non-GAP domains (Epstein lab).

The Epstein laboratory has submitted a manuscript describing our findings to *Cell Reports* and is currently under revision. We expect to resubmit within the next few months. The manuscript summarizes our findings that mutant larval zebrafish have dramatic learning and memory defects that can be markedly improved by adding appropriate chemicals (drugs) to the water. The findings rely upon a simple assay in which fish are habituated to an acoustic or visual stimulus (a loud noise or a moment of darkness). At first, the fish will respond to the stimulus with a characteristic bending and movement. However, after repeated stimuli, the fish habituate, and respond more slowly or not at all. Interestingly, *nf1a/b* mutant fish habituate (learn) poorly, but this poor learning behavior can be improved by drugs that elevate cAMP. If fish are once again tested after a period of an hour or more, trained fish "remember" the past experience and respond more slowly to renewed stimuli. Mutant fish lacking either 3 or 4 of the *nf1a/b* alleles have poor memory in this assay. This impaired memory phenotype can be improved with drugs that block ras or mapk (but not cAMP).

These results have several important implications for learning and memory deficits in humans with NF1, if the fish prove to be good models of the human disease. First, the defects are not "hard-wired" or developmental abnormalities, but rather appear to be functional defects that can be modified by chemicals. (We treated the fish for only 30 minutes before testing). This provides reason for hope that proper medications will be beneficial. Second, these findings suggest that targeting ras, as most therapeutic strategies to date have done, may not be sufficient and that learning and memory

defects can be individually improved by targeting cAMP and ras, respectively. In the future, it will be important to determine if these findings can be reproduced in mouse models, and if they predict successful treatments for learning disorders in humans with NF1.

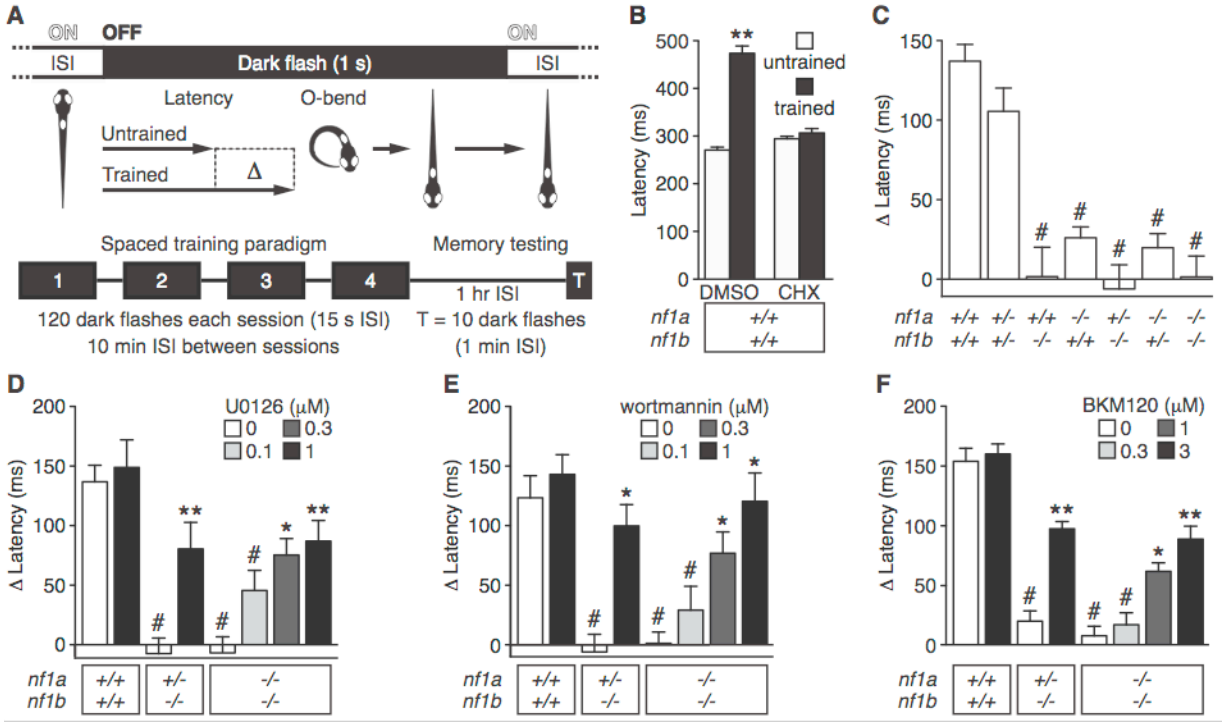


Figure 1. *nf1*-Mutant Larvae Exhibit Reduced Memory Recall
(A) Schematic representation of visual memory assay. ISI: interstimulus interval. (B-F) Mean O-bend latency (B) or latency change (C-F) 1 h after spaced training (test) versus untrained controls (n = 26 to 130 O-bend maneuvers per genotype/treatment). #P < 0.001 versus wild-type untreated (C) or DMSO-treated (B, D-F). *P < 0.01, **P < 0.001 versus same genotype, DMSO-treated. One-way ANOVA. Error bars denote SEM.

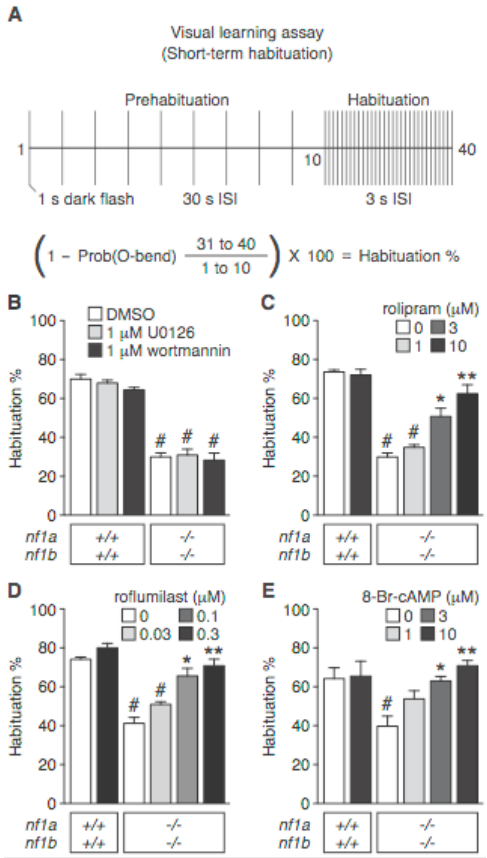
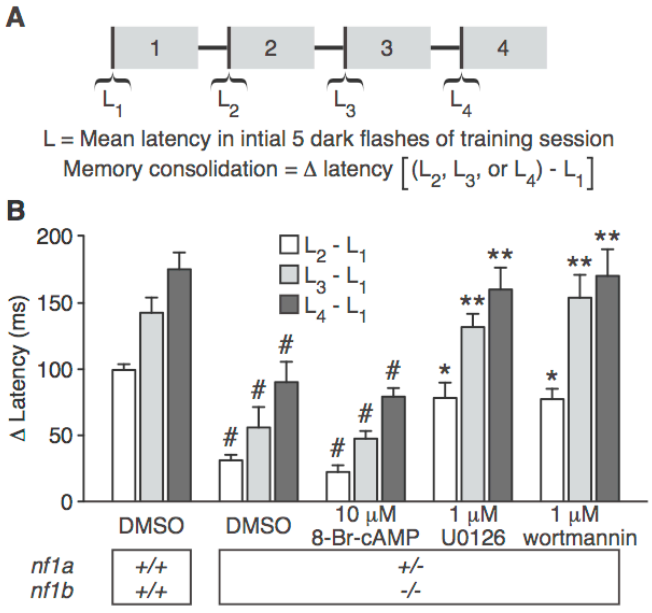


Figure 3. Inhibition of Ras Signaling Improves Memory Consolidation Deficits in *nf1* Mutants

(A) Schematic representation of visual memory consolidation measurement. (B) Mean O-bend latency change comparing responses to dark flash stimuli 1-5 of sessions 2-4 versus stimuli 1-5 of session 1 (*n* = 30 to 139 O-bend maneuvers per genotype/treatment). #*P* < 0.001 versus DMSO-treated wild-type larvae. **P* < 0.01, ***P* < 0.001 versus DMSO-treated *nf1a*$^{+/-}$; *nf1b*$^{-/-}$ larvae. One-way ANOVA. Error bars denote SEM.

Figure 2. cAMP Signaling Mediates *nf1*-Dependent Visual Learning

(A) Schematic representation of visual learning assay. ISI: interstimulus interval. (B-E) Mean habituation percentage to repeated dark flash stimulation (*n* = 3 groups of 15-20 larvae for all genotype/treatment groups). #*P* < 0.001 versus DMSO-treated wild-type larvae. **P* < 0.01, ***P* < 0.001 versus DMSO-treated *nf1a*$^{-/-}$; *nf1b*$^{-/-}$ larvae. One-way ANOVA. Error bars denote SEM.



Aim 2: To analyze the contributions of Nf1 GAP and other functional domains, based on Nf1 structure function analysis in Aim 1, to the suppression of malignant glioblastomas (CNS) and MPNSTs (PNS), which we have shown to develop in conjunction with p53 loss (Look lab).

The Look lab has continued to analyze the consequence of loss of NF1 in malignant glioblastomas and MPNSTs in vivo. To improve monitoring tumor initiation and progression in vivo in the *nf1/p53* mutant zebrafish lines, we have crossed the *nf1/p53* mutant fish to a sox10:EGFP line (sox10-expressing cells are labeled with GFP), as sox10 is reported to be consistently expressed in schwannian tumors (*3, 4*). As shown in Figure 4, the GFP driven by the sox10 promoter is highly expressed in the high-grade gliomas and MPNSTs, but not in the non-malignant neural crest-derived tissues, in the *nf1a+/-;nf1b-/-;p53-/-;sox10:GFP* adult zebrafish. This result indicates that *sox10* is highly expressed in high-grade gliomas and MPNSTs in our zebrafish model of type I neurofibromatosis.

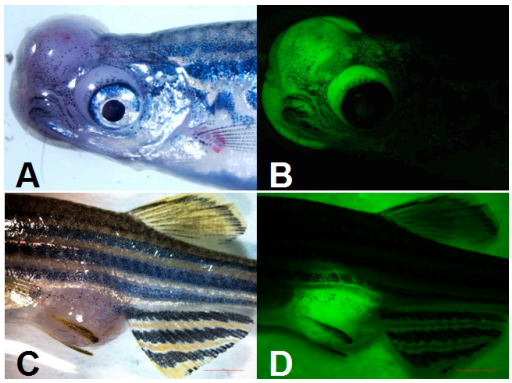


Figure 4. Sox10 is highly expressed in the high-grade gliomas and MPNSTs developed in the *nf1* mutant zebrafish. The initiation and progression of high-grade glioma (A, white light; B, GFP) and MPNST (C, white light; D, GFP) can be monitored in vivo in the *nf1* mutant zebrafish lines using the sox10:GFP fluorescent reporter.

Initial structure-function studies of NF1 will be performed using the human NF1 gene for transgenic rescue experiments. As *sox10* is highly expressed in high-grade gliomas and MPNSTs in our zebrafish model of type I neurofibromatosis (Figure 4), we will co-inject sox10:mcherry plus the sox10:NF1 mutant cDNAs to see which protein domains and amino acids of this very large protein are required for tumor suppression. The first domain that we will test is the GRD, which encodes the GAP function of NF1. We received cDNA coding the wild-type GRD domain of the human NF1 (GRDwt), as well as the GRD domain with an inactivating point mutation (R1276P, refer as GRDmut), from the Epstein lab, and sub-cloned the GRD domains downstream of the zebrafish sox10 promoter. Sox10:GRDwt or sox10:GRDmut was co-injected with a sox10:mCherry construct into the progeny of the *nf1a+/-;nf1b-/-;p53-/-;sox10:EGFP* zebrafish (Figure 5). We are currently waiting for the injectants to grow into adulthood to outcross for transgenic founders that have stably integrated the transgenes into the germline.

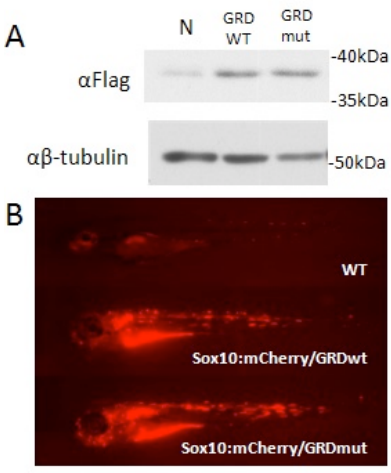


Figure 5. **Development of stable *nf1* mutant zebrafish lines with expression of the GRD domain of human NF1.**
(A) Western blot shows that Flag-tagged GRDwt or GRDmut is expressed in embryos at 5 days after injection.
(B) Mosaic expression of sox10:mCherry;sox10:GRD in injected embryos.

Previously, the earliest gliomas developed in the *nf1a+/-;nf1b-/-;p53-/-* line were observed from 19 weeks, and the earliest MPNSTs were observed after 20 weeks. To improve the model for the nf1 structure-function analysis using the NF1 constructs from Aim 1, the Look lab has continued with a panel of three independent strategies to further accelerate the tumor onset and increase tumor penetrance over the past year.

i. Introducing loss of pten into the *nf1/p53* mutant lines. Pten (phosphatase and tensin homolog) is one of the most frequently mutated or deleted tumor suppressors in human cancer. Mutations and homozygous deletions of pten were found in 36% of glioblastomas (GBM), the most malignant subtype of glioma (*5*). Loss or decrease of pten expression was also detected in a majority of human NF1-associated MPNST lesions (*6*). There are two orthologues of human pten in the zebrafish genome, namely ptena and ptenb (*7*). We obtained the loss-of-function *pten* mutant from Dr. Jeroen den Hertog and bred them with our *nf1a+/-;nf1b-/-;p53-/-* fish to establish a *nf1a+/-;nf1b+/-;p53+/-;ptena+/-;ptenb+/-* line. By incrossing the *nf1a+/-;nf1b+/-;p53+/-;ptena+/-;ptenb+/-* line, we obtained all possible combinational loss of nf1, p53 and pten in our zebrafish model. Accelerated formation of tumors associated with neurofibromatosis type 1 was observed in *nf1a+/-;nf1b-/-;p53-/-;ptena+/+;ptenb-/-* and *nf1a+/-;nf1b-/-;p53-/-;ptena+/-;ptenb-/-* lines. We are currently analyzing the tumor histology and establishing the *nf1a+/-;nf1b-/-;p53-/-;ptena+/+;ptenb-/-;sox10:GFP* and *nf1a+/-;nf1b-/-;p53-/-;ptena+/-;ptenb-/-;sox10:GFP* lines for GFP-based tumor watch and the structure-function studies using the NF1 constructs from Aim 1.

ii. Introducing human PDGFR into the *nf1a+/-;nf1b-/-;p53-/-* mutant line. PDGFR is frequently mutated in human GBM and MPNST *(3-6)*. We have obtained constructs of wild-type human PDGFR (PDGFRwt), as well as a constitutively active PDGFR mutant (PDGFRmut), from Dr. Eric Holland and sub-cloned the PDGFRwt and PDGFRmut downstream of the zebrafish sox10 promoter. Sox10:PDGFRwt or sox10:PDGFRmut was co-injected with a sox10:mCherry construct into the progeny of the *nf1a+/-;nf1b-/-;p53-/-;sox10:EGFP* zebrafish. Three months later, the primary injectants were outcrossed to indentify the minority of F1 animals that have stably integrated the transgenes into the germline. So far, we have established two independent *nf1a+/-;nf1b-/-;p53-/-;sox10:EGFP;sox10:PDGFRwt;sox10:mCherry* stable lines and one *nf1a+/-;nf1b-/-;p53-/-;sox10:EGFP;sox10:PDGFRmut;sox10:mCherry* stable line (Figure 6). We are currently performing a weekly tumor watch to determine the tumor spectrum in these lines and will select a best line for the NF1 structure-function studies.

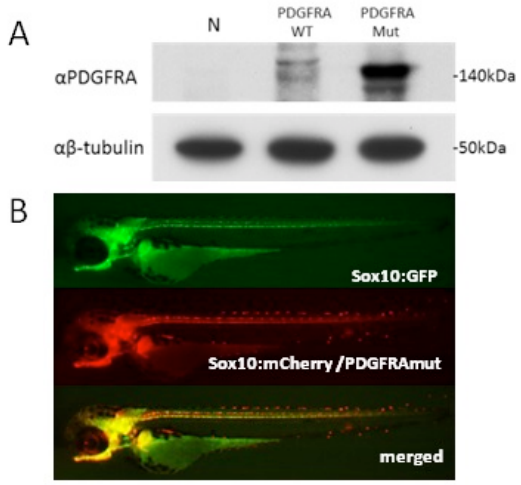


Figure 6. Development of stable *nf1* mutant zebrafish lines with expression of human PDGFR.

(A) Western blot shows that human PDGFRwt or PDGFRmut is expressed in *nf1a+/-;nf1b-/-;p53-/-;sox10:EGFP* embryos at 5 days after injection.

(B) A *nf1a+/-;nf1b-/-;p53-/-;sox10:EGFP;sox10:PDGFRmut;sox10:mCherry* stable line derived from the injections. Sox10:PDGFRmut and sox10:mCherry co-integrate together as they were co-injected into the one-cell fertilized zebrafish embryo, and show same expression pattern as sox10:GFP.

iii. Introducing mutations in H3F3A and ATRX into the *nf1a+/-;nf1b-/-;p53-/-* mutant line. It is recently found that H3F3A, ATRX, p53 and nf1 are the most frequently mutated genes in pediatric GBM (*7*). H3F3A encodes the replication-independent histone 3 variant H3.3 and ATRX (a-thalassaemia/mental retardation syndrome X-linked) is a chromatin remodeling factor required for H3.3 incorporation at pericentric heterochromatin and telomeres (*7*). The H3.3/ATRX perturbation was suggested to play a central role in pediatric GBM. We are applying an emerging genome editing technology called CRISPR-Cas (*8, 9*) to incorporate the deficiencies of the H3F3A and ATRX genes, as found in human patients, into the *nf1a+/-;nf1b-/-;p53-/-;sox10:GFP* zebrafish. We have injected CRISPR-Cas to knock-down ATRX, and are designing constructs to knock-in H3F3A K27M mutation. We expect that the incorporation of H3F3A and ATRX mutations will enhance high grade glioma development in our zebrafish lines. Once established, we will use the NF1 constructs from Aim 1 to perform nf1 structure-function analysis in these zebrafish to dissect the role of NF1 in pediatric high-grade gliomas associated with neurofibromatosis type 1.

KEY RESEARCH ACCOMPLISHMENTS

- Characterization of learning defects in *nf1* mutant zebrafish
- Characterization of memory defects in *nf1* mutant zebrafish
- Preparation of NF1 constructs for the structure-function analysis
- Generation of *nf1/p53/pten* mutant zebrafish
- Preparation of glioma/MPNST models for the structure-function analysis

REPORTABLE OUTCOMES

- Abstract: In vivo Analysis of the Consequences of loss of the NF1 Tumor Suppressor in High-Grade Glioma, Malignant Peripheral Nerve Sheath Tumor (MPNST) and Neuroblastoma. Shuning He. NF Conference 2014. Washington DC, June 7-10 2014.
- Abstract: In vivo Analysis of the Consequences of loss of the NF1 Tumor Suppressor in High-Grade Glioma, Malignant Peripheral Nerve Sheath Tumor (MPNST) and Neuroblastoma. Shuning He. 11th International Conference on Zebrafish Development and Genetics. Madison, WI, June 24-28 2014
- Abstract: In vivo Analysis of the Consequences of loss of the NF1 Tumor Suppressor in High-Grade Glioma, Malignant Peripheral Nerve Sheath Tumor (MPNST) and Neuroblastoma. Shuning He. Zebrafish Disease Models Conference. Madison, WI, June 28- July 1 2014
- Presentation: Dr. Look presented the Zebrafish Models of Pediatric and Adult High Grade Gliomas on the Neuro-Oncology Multidisciplinary Conference in Dana-Farber Cancer Institute/ Brigham and Women Hospital (Janurary 10, 2014)
- Presentation: Dr. Epstein recently delivered a prestigious keynote address at the Washington University of St. Louis NF Center Research Symposium (May 16, 2014).
- Employment or research opportunities applied for and/or received based on experience/training supported by this award: Arun Padmanabhan, who performed critical work for this project, has accepted a position as a medical resident at the Massachusetts General Hospital and is highly likely to continue in a career as a physician-scientist. This grant and project played a major role in

shaping his career and in providing the training necessary to obtain one of the most coveted medical residency positions in the country.

- Employment or research opportunities applied for and/or received based on experience/training supported by this award: Dr. Dong Hyuk Ki was recruited from Development biology of Stony Brook University to the Look lab to learn about neurofibromatosis type I and to carry out the studies on new pathways for targeted therapy of NF1-associated MPNST through in vivo studies in the zebrafish model system, based on this award.
- Employment or research opportunities applied for and/or received based on experience/training supported by this award: Dr. Felix Oppel was recruited from Development biology of Stony Brook University to the Look lab to learn about neurofibromatosis type I and to carry out the studies on the role of recurrent mutations in ATRX and H3F3A in pediatric high-grade gliomas, based on this award.

CONCLUSION

During the past year, we have continued to further our studies towards meeting the goals set forth in the funded proposal. An exciting phenotype has been identified that is relevant to the human NF1 disease and to the potential development of meaningful therapies. In collaboration with the Granato lab at Penn, the Epstein lab has discovered striking learning and memory defects in mutant fish. In the Look lab, the zebrafish models of the NF1 tumor suppressor linked cancers of MPNST and glioma have been completely updated to make them optimal for the structure function studies. Three different strategies are being applied to further accelerate the onset of these tumors to increase the precision of the structure function studies to elucidate the mechanism of tumor suppression by NF1. We have shipped our NF1 zebrafish lines to colleagues in the NF1 and zebrafish communities. These genetically engineered lines will be excellent candidates for use in screening small molecules and for use in performing genetic screens to identify modulators of this important disease.

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APPENDICES

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, study questionnaires, and surveys, etc.

Abstract: In vivo Analysis of the Consequences of loss of the NF1 Tumor Suppressor in High-Grade Glioma, Malignant Peripheral Nerve Sheath Tumor (MPNST) and Neuroblastoma. Shuning He.

In vivo Analysis of the Consequences of loss of the NF1 Tumor Suppressor in High-Grade Glioma, Malignant Peripheral Nerve Sheath Tumor (MPNST) and Neuroblastoma

Shuning He Ph.D., *Dana-Farber Cancer Institute*

Type 1 Neurofibromatosis (NF1) is caused by mutations in the *NF1* gene, which encodes neurofibromin, a protein of over 4,000 amino acids. This large protein is expressed widely during embryonic development and in the adult and functions as a prominent tumor suppressor. Patients with loss of NF1 are prone to develop MPNST and both low and high grade glioma, and other tumors such as neuroblastoma have somatically acquired loss of *NF1*. While the majority of therapeutic approaches have targeted the Ras GAP-related domain (GRD) of neurofibromin, numerous mutations outside the GRD, which do not appear to affect protein stability or GAP function, also render NF1 patients prone to develop tumors. Evolutionary conservation of domains outside of GRD also suggests that additional important functions are yet to be discovered, especially regarding tumor suppression. In this study, we hope to capitalize on the advantages of zebrafish as a model system to identify unrecognized functional domains appropriate for therapeutic targeting and to address pressing questions relevant to the generation of new and effective therapies for NF1. We have identified two separate zebrafish *nf1* genes and used a zinc-finger nuclease strategy to generate multiple loss-of-function *nf1* mutant zebrafish lines. Loss of *nf1* contributes to tumorigenesis as demonstrated by an accelerated onset and increased penetrance of high-grade gliomas and MPNSTs in zebrafish, in combination with loss-of-function mutations of p53. Loss of *nf1* also greatly accelerated onset of neuroblastoma induced by MYCN overexpression, with a penetrance of nearly one hundred percent by 5 weeks. We are applying these zebrafish tumor models to analyze the contribution of the NF1 GRD domain to tumor suppression, and to discover other novel functional domains of NF1 that can cooperate with the GRD in NF1-mediated tumor suppression. We hope to develop models suitable for small molecule drug screens, conducted in zebrafish embryos, to identify new drugs for high-grade gliomas, MPNSTs and neuroblastomas with loss of nf1 function.

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Shizhen Zhu MD Ph.D., Dana-Farber Cancer Institute
Eric D. de Groh Ph.D., University of Pennsylvania
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